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Mutant Poly (ADP-Ribose) Polymerase

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12a. DISTRIBUTION / AVAILABILITY STATEMENT

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During the first year of funding we have developed a recombinant plasmid (pPSA (e/p)-DBD/F) comprised of the coding region of the PARP-DBD linked to 5'-flanking sequences (1.3 kb upstream enhancer/ 0.6 kb minimal promoter) of the human PSA gene. The present study reports the development and characterization of LNCaP prostate carcinoma cell sublines expressing the human PARP-DBD protein in constitutive and androgen-inducible fashion. Tissue specificity of PARP-DBD expression in human tumor cells was confirmed using the PSA-positive (LNCaP) and PSA-negative (PC-3) prostate cancer cells and cells of non-prostate origin, Ewing's sarcoma (A4573 cells). We found that exposure of LNCaP cells stably transfected with pPSA (e/p)-DBD/F to synthetic androgen (R1881) resulted in dose-dependent stimulation of PARP-DBD expression at levels of mRNA and protein. Androgen-dependent fashion of PARP-DBD expression in LNCaP cells was further confirmed by *in situ* immunodetection of DBD-Flag fusion protein using fluorescence microscopy. Established cell lines provide a convenient experimental model to study effects of the PARP-DBD expression on prostate tumor responses to ionizing radiation and genotoxic drugs.

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INTRODUCTION

The central objective of the proposal is to express the DNA-binding domain of PARP under control of prostate tissue-specific promoter in prostate cancer cells and sensitize them to radiotherapy or chemotherapy. We hypothesize that the sustained presence of the PARP-DBD in prostate tumor tissue will kill cells via apoptosis in response to massive DNA damage induced by ionizing radiation or genotoxic drugs. To test this hypothesis we will utilize the prostate-specific antigen (PSA) promoter to direct the PARP-DBD expression to prostate cancer cells. Using PSA-producing cells (LNCaP) and cells that do not express PSA (PC-3) as the primary experimental model system we propose the experimental approach designed to: 1) produce prostate carcinoma cell sublines which allow androgen-inducible, high-level expression of the PARP-DBD and 2) test the DNA-binding domain of PARP as a molecular sensitizer for improving responses of prostate tumor cells to gamma radiation and DNA-damaging drugs. The completion of experiments proposed in this project will contribute to the development of complementary biotherapeutic approaches in the treatment of prostate cancers, which fail local-regional therapy.

In the following sections, we describe the progress we have made in each strategy during the last budget year.

ANNUAL REPORT

I. ORIGINAL STATEMENT OF WORK

The proposed studies are designed to explore the potential of novel combination therapy that would utilize the tissue-specific (prostate) and radiation-specific (damages in DNA) gene therapy for prostate cancer.

Task 1. To establish prostate cancer cell lines stably expressing PARP-DBD under control of PSA promoter regulatory elements (months 1-19)

- i. develop a series of plasmids to drive prostate tissue-specific expression of PARP-DBD gene (months 1-8)
- ii. produce PARP-DBD expressing sublines from LNCaP prostate carcinoma cell line (months 9-13)
- iii. test tissue-specificity and responsiveness of PARP-DBD expression to androgens (months 14-19)

Task 2. To investigate the potential of PARP-DBD protein for sensitization of prostate cancer cells to ionizing radiation and DNA-damaging drugs (months 19-36)

- i. test the PARP-DBD expression levels for efficiency to inhibit PARP activity and DNA damage repair following gamma radiation and drug treatments (months 19-24)
- ii. investigate the effects of PARP-DBD expression on cell viability, cycle progression and apoptosis induction post-irradiation (months 24-31)

- iii. determine whether cell sensitization by PARP-DBD depends upon the type of DNA damage inflicted on the cells (months 26-32)
- iv. conduct radiation survival curve analysis on prostate cancer cell lines expressing differential levels of PARP-DBD to assess its radiosensitizing ability (months 28-36)

II. RESEARCH ACCOMPLISHED

Task 1.

PARP-DBD expression in PSA-producing and PSA-negative human tumor cells

During the first year of funding, we constructed a plasmid pCMV-DBD/F, which permits constitutive expression of human PARP-DBD under control of the CMV promoter, and a plasmid, pPSA(e/p)-DBD/F for expression of the human PARP-DBD in androgen-inducible and PSA-dependent fashion. In this study, the constructs were used to generate PARP-DBD expressing sublines from LNCaP prostate carcinoma cell line and to test tissue-specificity and responsiveness of PARP-DBD expression to androgens in these cells.

To determine whether PARP-DBD can be expressed in mammalian cells, prostate carcinoma cell lines LNCaP and PC-3 and Ewing's sarcoma (A4573 cell line) cells were transiently transfected with the pCMV-DBD/F plasmid that allow constitutive expression of PARP-DBD. Figure 1 shows that expression of PARP-DBD-Flag fusion proteins can be reliably detected by Western immunoblotting in all tested cell lines for at least 48 hours post-transfection.

Tissue specificity of PARP-DBD expression under control of PSA promoter/enhancer was evaluated in transient transfection assays using the PSA-positive (LNCaP) and PSA-negative (PC-3) prostate cancer cells and cells of non-prostate origin such as Ewing's sarcoma (A4573 cell line). We found that PSA enhancer/promoter driven expression of the human PARP-DBD was observed only in PSA-producing LNCaP prostate carcinoma cells but not in PSA-independent cell lines, at least at immunodetectable levels (Fig. 1).

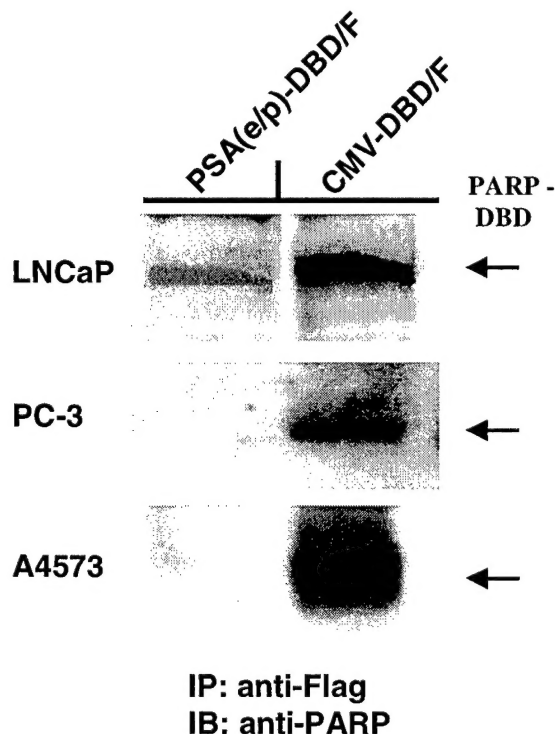


Figure 1: PARP-DBD expression in PSA-producing and PSA-negative cells. PSA-positive (LNCaP), PSA-insensitive (PC-3) prostate cell lines, and non-prostate (Ewing's sarcoma, A4573 cell line) cells were transiently transfected with pPSA(EP)-DBD/F or pCMV-DBD/F. Cells were harvested 48 hours after transfection and PARP-DBD expression was immunodetected.

To confirm functional activity of the PARP-DBD expressed in LNCaP cells, we performed DNA binding assays (Fig. 2). PARP affinity for DNA was assayed using double-stranded oligonucleotides coated onto magnetic beads. Briefly, 100 μ g of streptavidin-coated Dynabeads (DynaL Biotech) were incubated with 120 pmoles of 5'-biotinylated double-stranded pJ α oligonucleotides (1) at room temperature for 30 min in accordance with manufacturer's instruction. Purified recombinant human PARP protein (Alexis, specific activity 30 U/ μ g) or crude cell lysate in IP buffer were combined with pJ α -affinity beads and incubated for 30 min with gentle agitation at room temperature. The protein-bound beads were separated using a magnetic separator (DynaL), bound proteins were eluted with 30 μ l of 1M NaCl and subsequently analyzed by Western blotting using goat polyclonal anti-PARP antibody (R&D Systems).

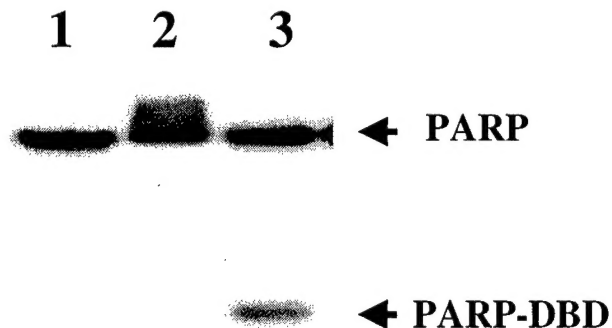


Figure 2: DNA binding activity of PARP and PARP-DBD proteins. 1, purified recombinant human PARP protein; 2, LNCaP cell lysate, 3, lysate made from LNCaP cells transfected with pCMV-DBD/F.

Establishment of stable transfected LNCaP cell lines.

LNCaP cells were transfected with pPSA (e/p) -DBD, pCMV-DBD or with control, neomycin-resistant expression vector p Δ CMV-DBD, respectively, using an activated-dendrimer reagent ("Superfect", Qiagen). Transfection medium was replaced with complete growth medium and cells were incubated for 48 h to allow expression of neomycin-resistance, followed by replating into selective medium containing 300 μ g/ml G418 (Geneticin; GIBCO). The G418-resistant colonies from each replicated experiment were pooled to form polyclonal cell populations and were routinely maintained in medium containing 300 μ g/ml G418. Established polyclonal LNCaP sublines were subsequently subjected to screening for androgen-dependent PARP-DBD expression (see below, Fig. 3 and Fig. 4).

Androgen-regulated expression of PARP-DBD under control of PSA promoter/enhancer in LNCaP cells.

LNCaP cells stably transfected with pPSA (e/p)-DBD/F (see example 6 for description) were grown in media containing charcoal-stripped serum for seven days followed by incubation for 24 hours in absence or in presence of synthetic androgen, R1881 (0-10 nM). Androgen-regulated expression of the human PARP-DBD in LNCaP cells was assessed by Western blot analysis (Fig. 3A) and by RT-PCR (Fig. 3B). Parental LNCaP cells and LNCaP cell subline (CMV-DBD) were used as negative and positive controls, respectively, for PARP-DBD expression. We found that exposure of

LNCaP cells stably transfected with pPSA (E/P)-DBD/F to androgen (R1881) resulted in dose-dependent stimulation of PARP-DBD expression at levels of mRNA and protein (Fig 3A and 3B).

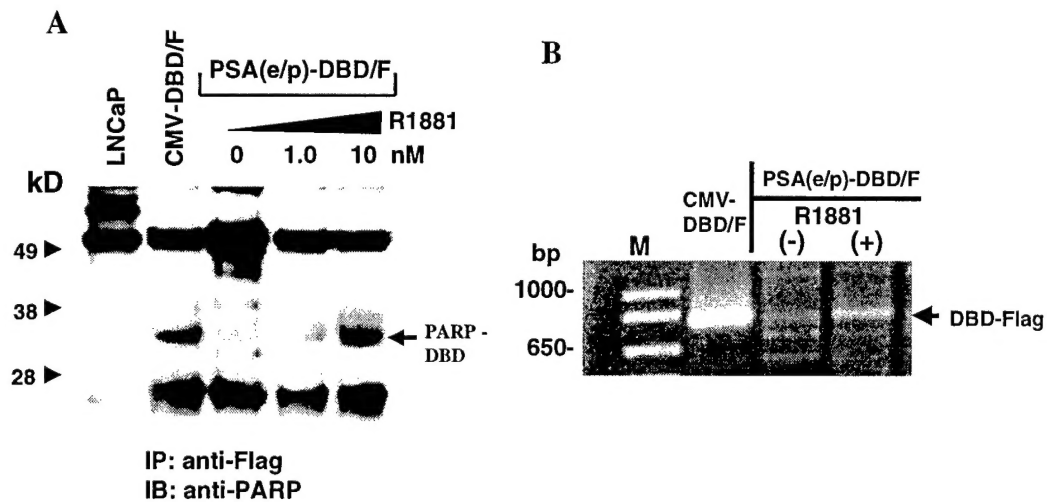


Figure 3: LNCaP cells were stably transfected with plasmid vectors that allow constitutive (pCMV-DBD/F) or androgen-inducible (pPSA-DBD/F) expression of PARP-DBD. The established cell sublines were analyzed for androgen-dependent induction of the PARP-DBD expression by Western blotting and RT-PCR. **A**, Immunodetection of PARP-DBD-Flag fusion protein in LNCaP cell sublines expressing PARP-DBD under control of CMV promoter (CMV-DBD) or PSA enhancer/promoter (PSA-DBD). LNCaP sublines transfected with pPSA-DBD/F were maintained in absence or in presence of synthetic androgen, R1881 (0-10 nM) as described in "Materials and Methods". Parental LNCaP cells were used as a negative control for PARP-DBD expression. The migration of the DBD-Flag fused protein is indicated on the right. **B**, RT-PCR analysis of mRNA for PARP-DBD-Flag fused protein. Specific RT-PCR product is indicated on the right, and molecular weight markers (M) are shown on the left.

Androgen-regulated expression of human PARP-DBD in these cells was further confirmed by *in situ* immunodetection of DBD-Flag fusion protein using fluorescence microscopy (Fig. 4).

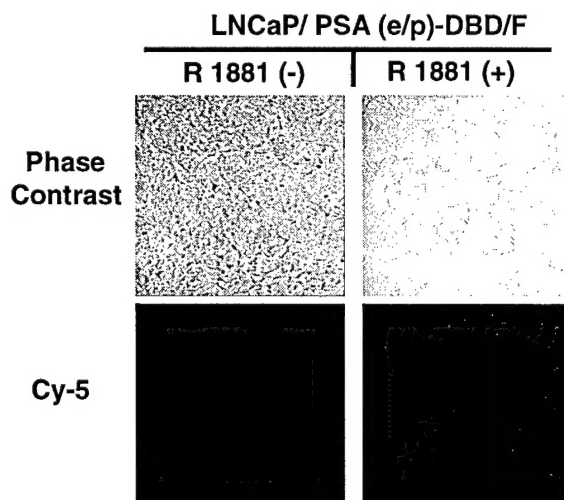


Figure 4: pPSA-DBD/F drives androgen-responsive expression of PARP-DBD in LNCaP cells. For *in situ* PARP-DBD immunodetection, LNCaP cells were grown in media containing 10% charcoal-stripped fetal bovine serum for seven days. Following induction of PARP-DBD expression by synthetic androgen R1881 (10nM) for 24 h, cells were immunostained for PARP-DBD-Flag fusion protein. Transmitted (phase contrast) and Cy5 (red fluorescence) images were acquired using IX 70 confocal laser scanning microscope (Olympus).

Task 2.**PARP-DBD and DNA-damage response.**

The study was initiated to investigate the role of PARP-DBD in cellular responses to ionizing radiation and DNA damaging treatments. Data from many studies show that PARP is involved in numerous biological functions, all of which are associated with breaking and rejoining DNA strands, and it plays a pivotal role in DNA damage repair (2). Furthermore, it was demonstrated that PARP-DBD irreversibly binds to broken DNA strands and make them inaccessible for DNA repair (3).

Recent studies have also implicated PARP in transcription of eukaryotic genes (9-16). To elucidate mechanistical basis for PARP role in transcription, we investigated whether PARP can be recruited to gene-regulating sequences and whether its DNA-binding activity has a role in PARP-mediated gene regulation. Based on the ability of PARP Based on PARP ability to interact with partially unwound DNA (4), we reasoned that DNA secondary structures with single-stranded character may provide potential binding sites for PARP in gene regulating sequences in the absence of DNA strand breaks. In this work we investigated the interactions between PARP protein and DNA structures of different complexity such as DNA heteroduplexes carrying stable secondary structures and superhelical DNA containing PARP promoter sequences. We found that PARP can recognize non-canonical conformations (hairpins) in a DNA end-independent fashion, and it is capable of *in vitro* binding to the PARP promoter sequences where the dyad symmetry elements may form the cruciforms. Using a chromatin cross-linking and immunoprecipitation assay we show that the human PARP promoter is an *in vivo* target for PARP protein. Further, we show that PARP protein down-regulates its gene promoter, and that DNA-binding activity of PARP is essential for its function in transcription (Fig. 5).

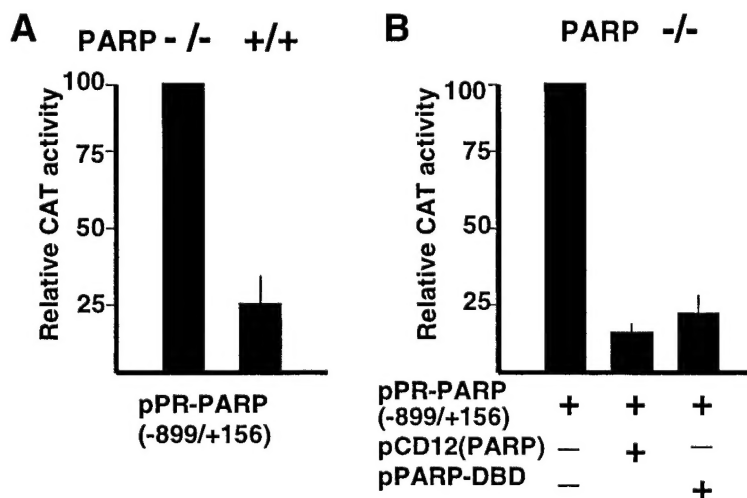


Figure 5. PARP protein is a transcriptional repressor. **A**, PARP promoter (pPR-PARP) transcriptional activity in wild-type (PARP ^{+/+}) and PARP ^{-/-} fibroblasts. **B**, expression of human PARP or its DNA-binding domain down-regulates promoter activity. PARP ^{-/-} cells were co-transfected with pPR-PARP and plasmids encoding for full length PARP (pCD12) or its truncated mutant (pPARP-

DBD/F). CAT activity of pPR-PARP in PARP^{-/-} cells was arbitrarily taken as 100%. Means of triplicate experiments normalized by co-transfected β -gal and standard deviations are indicated. Experimental procedures for these experiments are described in manuscript (J. Biol. Chem., 2002, 277: 665-670) included in **appendix**.

Our data suggest that a hierarchy of PARP function may exist under which transcriptional repression may be abrogated in response to DNA damage due to a higher affinity of PARP for DNA breaks and its dissociation from DNA following protein automodification (Fig. 6). This concept integrates PARP functions in DNA repair (a nick-protection mechanism) (2) and in transcriptional control of gene(s) involved in immediate cellular response to ionizing radiation and DNA damaging drugs. Although the evidence supporting such a mechanism is not yet available, it is conceivable that the sharing of components such as PARP by DNA repair and transcription allows both events to control cellular survival in response to ionizing radiation and DNA-damaging treatments.

Currently we perform experiments to assess the effects of the PARP-DBD expression in LNCaP cells on their sensitivity to ionizing radiation and DNA damaging treatments

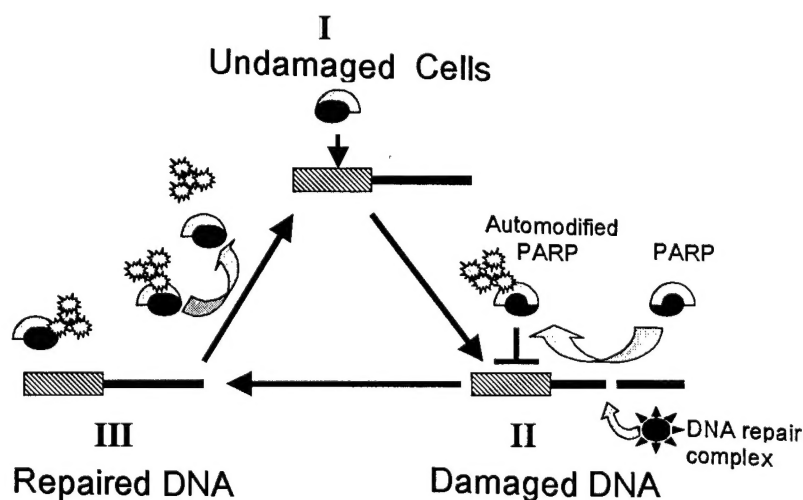


Figure 6. A model for PARP-mediated regulation of transcription. I, in undamaged cells, unmodified PARP molecules bind to the DNA secondary structures within the gene promoter (denoted by a striped box). Such macromolecular interactions between PARP protein and a promoter region constitute a repressor function for PARP in transcription. II, in response to DNA damage, PARP binding to the DNA ends triggers its catalytic activity. Subsequent poly(ADP-ribose)ation of free and bound PARP in the presence of intracellular NAD⁺ prevents its interaction with the promoter regions. This alleviates the PARP-mediated block on the promoter and up-regulate transcription of its own and other genes involved in the DNA damage response. III, the DNA-binding activity of PARP is restored following DNA damage repair and the degradation of the ADP-ribose polymers by poly(ADP-ribose) glycohydrolase leading to re-assembly of PARP-promoter complexes and inhibition of transcription.

EXPERIMENTAL PROCEDURES

Cell lines and tissue culture. The androgen responsive prostate carcinoma LNCaP and androgen independent PC-3 cell lines were obtained from American Type Culture Collection (Manassas, VA) and maintained by serial passage in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in an atmosphere of 5% CO₂ in air. Cells subjected to androgen stimulation tests were maintained in media with 10% charcoal-stripped fetal bovine serum for seven days before the addition of synthetic androgen R1881 (Perkin Elmer Life Science). Ewing's sarcoma cell line A4573 (kindly provided by Dr. T. Kinsella, University of Wisconsin, Madison) were maintained in Eagle's minimal essential medium (GIBCO) as described (5). All irradiations were performed at room temperature, in air, using a ¹³⁷Cs source in a "JL Shepard MARK I" laboratory irradiator at a dose rate of 3.85 Gy/min.

RT-PCR Analysis. RNA was isolated from cells using TRIzol Reagent (Gibco) according to manufacturer's protocol. The primers for human DBD-Flag fusion protein were sense, 5' -ATCACCATCACCATCA-3' and antisense, 5' -CCTTTATCGTCATCGT-3'. RT-PCR was performed using 2 µg of total cellular RNA and the ThermoScript RT-PCR System (Gibco). The PCR amplification was carried out in a Perkin-Elmer amplification cyclor (Wellesley, MA) during 35 cycles with denaturing at 96°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 1 min. The amplified RT-PCR products were analyzed by 1% agarose gel electrophoresis, visualized by ethidium bromide staining and photographed under UV illumination.

DNA transfections. DNA transfections were carried out using an activated-dendrimer reagent ("Superfect", Qiagen). Briefly, cells (2.0 x 10⁵) were plated into 60 mm tissue culture dishes coated with poly-L-lysine (Sigma) and maintained in culture for 2 days. Transfections were performed with 5 µg of pCMV-DBD or pPSA-DBD plasmids using a ratio of DNA to "Superfect" reagent of 1:10. Cells were harvested 48 h after transfection for assays of PARP-DBD expression. Stable transfection of human prostatic adenocarcinoma cell line LNCaP and clonal selection were performed as previously described (5).

PARP-DBD immunodetection. Immunodetection of PARP-DBD Flag-fusion protein in human prostate carcinoma cells was performed as previously described (6). In brief, logarithmically growing cells were washed twice with cold PBS and lysed at 4°C for 30 min in buffer: 0.5% Triton X-100, 0.5% NP-40, 2mM NaOAc, 150 mM NaCl, 2mM EDTA, 50mM Tris-HCl (pH=7.5), 1 mM phenylmethylsulfonyl fluoride, 20 µg /ml aprotinin and 20 µg/ml leupeptin. Insoluble material was removed by centrifugation at 4°C for 30 min at 16,000x g and protein concentrations were determined using the "Micro BCA protein assay" (Pierce). Immunoprecipitation was performed by incubating the lysate with anti-Flag M2 monoclonal antibody agarose affinity gel (Sigma). Immune complexes were washed 6 times with 100mM Tris (pH 7.5) - 0.5% Tween buffer and subsequently resolved on NuPAGE Novex 4-12% gradient Bis-Tris gels (Invitrogen), followed by Western blotting using polyclonal anti-PARP antibody (R&D System; dilution 1:1,000) directed against the aa 71-329 of PARP protein. The secondary antibody was donkey anti-goat IgG (dilution 1: 2,000) conjugated to horseradish peroxidase (Santa Cruz). Signals were detected using the enhanced chemiluminescence system (Amersham). In some experiments, western immunoblot analyses for PARP were performed

using rabbit polyclonal antibody (Cell Signaling, #9542) with a synthetic peptide (KLH coupled) corresponding to the caspase cleavage site in PARP (dilution 1:2000).

Immunofluorescence and phase contrast microscopy. For *in situ* PARP-DBD immunodetection, LNCaP cells were grown on poly-D-lysine-treated slides (Fisher Scientific) in media containing 10% charcoal-stripped fetal bovine serum for seven days. Following induction of PARP-DBD expression by synthetic androgen R1881 (10nM) for 24 h, medium was removed and cells were subjected to fixation with 3.7% paraformaldehyde for 10 min as described (7). After rehydration in PBS, cells were permeabilized with PBS-0.2 % Triton X-100 for 10 min, washed with PBS and incubated for 30 min with anti-Flag M2 monoclonal antibody (Sigma; dilution 1:200). Washes were followed by 30 min incubation with Cy-5 conjugated secondary antibody (Jackson ImmunoResearch, dilution 1:200) in PBS, contained 10% Donkey serum, 0.1% 300 Bloomgelatin. Slides were then washed with PBS, blotted dry and mounted with glass cover slips using a "Prolong Antifade " mounting medium (Molecular Probes, Inc). Confocal images (transmitted and Cy5 fluorescence) were acquired using IX 70 confocal laser scanning microscope (Olympus, Melville, NY).

KEY RESEARCH ACCOMPLISHMENTS

- LNCaP cell subline stably expressing functionally active PARP-DBD under control of CMV promoter was developed.
- LNCaP cell subline stably expressing PARP-DBD under control of PSA promoter regulatory elements was developed.
- We demonstrated that PSA promoter driven PARP-DBD expression in LNCaP cells shows tissue-specificity and responsiveness to androgens.
- We demonstrated that DNA-binding activity of PARP is essential for its function in regulation of gene expression, and that PARP-DBD may interfere with PARP-mediated regulation of transcription.

REPORTABLE OUTCOMES

Manuscripts:

Soldatenkov VA, Chasovskikh S, Potaman VN, Trofimova I, Smulson ME, Dritschilo A: Transcriptional repression by binding of poly(ADP-ribose) polymerase to promoter sequences. *J. Biol. Chem.*, 277: 665-670, 2002

Papers presented:

Trofimova I, McDermott F, Dritschilo A, Notario V, **Soldatenkov VA**. Down-regulation of ETS1 transcription factor inhibits apoptosis of Ewing's tumor cells. 92nd An Mtg AACR, New Orleans, LA. Proceedings, p. 637, 2001

Soldatenkov VA, Chasovskikh S, Potaman VN, Dritschilo A. Transcriptional autoregulation of the human poly(ADP-ribose) polymerase gene. 48th An Mtg Radiat. Res. Soc. San Juan, Puerto Rico, p.141-142, 2001

Trofimova I, Dritschilo A, **Soldatenkov VA**. Poly(ADP-ribose) polymerase and transcriptional regulation in DNA-damage response. Proceedings of IIAR Conference (Athens, Greece). *Anticancer Research*, 21:1535-1536, 2001

Cell lines developed:

- LNCaP/CMV-DBD, LNCaP cells stably transfected with pCMV-DBD/F
- LNCaP/PSA(e/p)-DBD, LNCaP cells stably transfected with pPSA(e/p)-DBD/F

CONCLUSIONS

The present study reports the development and characterization of LNCaP prostate carcinoma cell sublines expressing the human PARP-DBD protein in constitutive and androgen-inducible fashion. Established cell lines provide a convenient experimental model to study effects of the PARP-DBD expression on prostate tumor responses to ionizing radiation and genotoxic drugs.

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APPENDIX

1. Reprint of Journal article: *J. Biol. Chem.*, 277: 665-670, 2002
2. FIGURE 4. (color images)

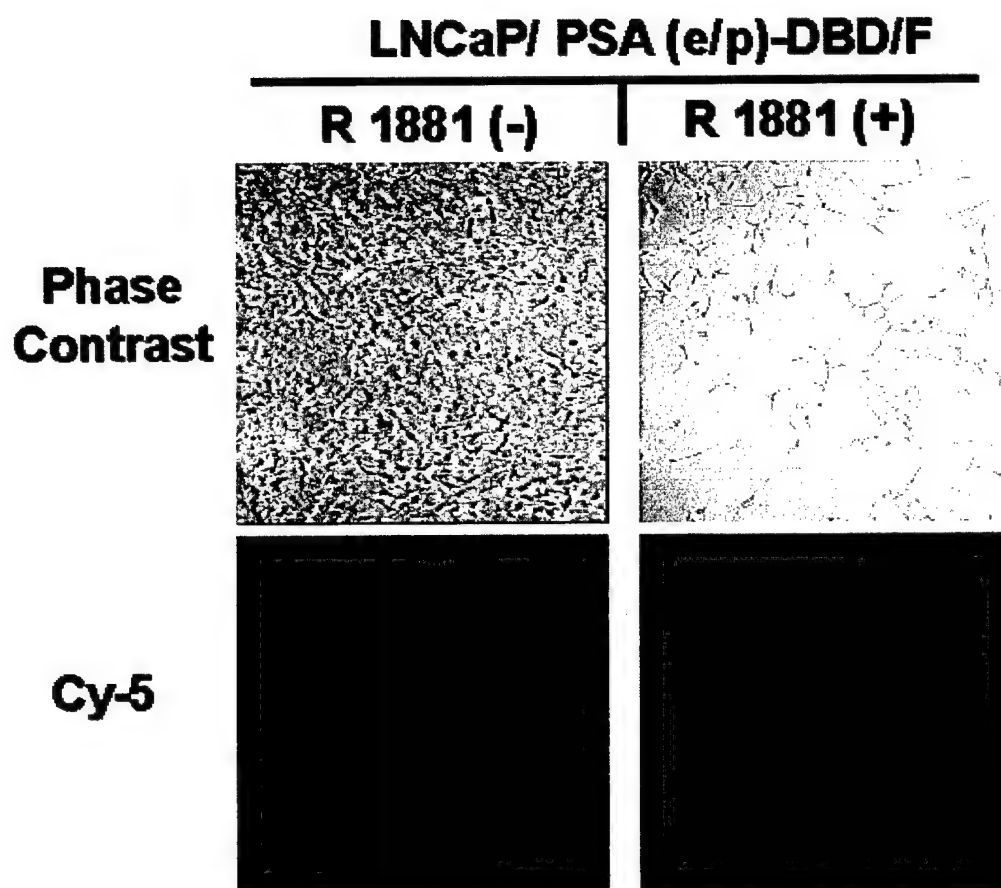


Figure 4: pPSA-DBD/F drives androgen-responsive expression of PARP-DBD in LNCaP cells. For *in situ* PARP-DBD immunodetection, LNCaP cells were grown in media containing 10% charcoal-stripped fetal bovine serum for seven days. Following induction of PARP-DBD expression by synthetic androgen R1881 (10nM) for 24 h, cells were immunostained for PARP-DBD-Flag fusion protein as described in "Materials and Methods". Transmitted (phase contrast) and Cy5 (red fluorescence) images were acquired using IX 70 confocal laser scanning microscope (Olympus).

Transcriptional Repression by Binding of Poly(ADP-ribose) Polymerase to Promoter Sequences*

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Poly(ADP-ribose) polymerase (PARP) is a DNA-binding enzyme that plays roles in response to DNA damage, apoptosis, and genetic stability. Recent evidence has implicated PARP in transcription of eukaryotic genes. However, the existing paradigm tying PARP function to the presence of DNA strand breaks does not provide a mechanism by which it may be recruited to gene-regulating domains in the absence of DNA damage. Here we report that PARP can bind to the DNA secondary structures (hairpins) in heteroduplex DNA in a DNA end-independent fashion and that automodification of PARP in the presence of NAD⁺ inhibited its hairpin binding activity. Atomic force microscopic images show that *in vitro* PARP protein has a preference for the promoter region of the PARP gene in superhelical DNA where the dyad symmetry elements likely form hairpins according to DNase probing. Using a chromatin cross-linking and immunoprecipitation assay we show that PARP protein binds to the chromosomal PARP promoter *in vivo*. Reporter gene assays have revealed that the transcriptional activity of the PARP promoter is 4–5-fold greater in PARP knockout cells than in wild type fibroblasts. Reintroduction of vectors expressing full-length PARP protein or its truncated mutant (DNA-binding domain retained but lacking catalytic activity) into PARP^{−/−} cells has conferred transcriptional down-regulation of the PARP gene promoter. These data provide support for PARP protein as a potent regulator of transcription including down-regulation of its own promoter.

Poly(ADP-ribose) polymerase (PARP,¹ EC 2.4.2.30) is a chromatin-associated enzyme that catalyzes the transfer of successive units of the ADP-ribose moiety from NAD⁺ to itself and other nuclear acceptor proteins (1). PARP is a zinc finger-containing protein, which allows enzyme binding to either double or single strand DNA breaks without any apparent se-

quence preference (2, 3). The catalytic activity of PARP is strictly dependent on the presence of strand breaks in DNA and is modulated by the level of automodification (4, 5). Data from many studies show that PARP is involved in numerous biological functions, all of which are associated with breaking and rejoining DNA strands, and it plays a pivotal role in DNA damage repair (2, 6–8).

Recent studies have implicated PARP in transcription of eukaryotic genes (9–16). PARP-dependent gene regulation involves poly(ADP-ribosyl)ation of transcription factors, which, in turn, prevents their binding to specific promoter sequences (10). The basal transcription factors TFIIF and TEF-1 as well as transcription factors TATA box-binding protein, YY1, SP-1, cAMP-response element-binding protein, p53, and NFκB are all highly specific substrates for poly(ADP-ribosyl)ation (10, 11, 14, 16). PARP may also interact directly with gene promoters. For instance, recombinant full-length PARP bound the DNA sequences within the MCAT1 regulatory element (11) and to the DF4 protein binding site of the *Pax-6* gene neuroretina-specific enhancer (17). Furthermore, PARP involvement in the active transcriptional DNA-protein complex formation on *Reg* promoter has been recently reported (12). Together these observations suggest that PARP may exert its function in transcription through direct binding to the gene-regulating sequences and through modification of transcription factors by poly(ADP-ribosyl)ation. However, total dependence of PARP function on DNA strand breaks (5) does not provide a mechanism by which it may ADP-ribosylate transcription regulators and be recruited to gene-regulating sequences in the absence of DNA damage.

Based on the ability of PARP to interact with partially unwound DNA (18, 19), we reasoned that DNA secondary structures with single-stranded character may provide potential binding sites for PARP in gene-regulating sequences in the absence of DNA strand breaks. In this work we investigated the interactions between PARP protein and DNA structures of different complexity such as DNA heteroduplexes carrying stable secondary structures and superhelical DNA containing PARP promoter sequences. We found that PARP can recognize noncanonical conformations (hairpins) in a DNA end-independent fashion, and it is capable of *in vitro* binding to the PARP promoter sequences where the dyad symmetry elements may form the cruciforms. Using a chromatin cross-linking and immunoprecipitation assay we show that the human PARP promoter is an *in vivo* target for PARP protein. Further, we show that PARP protein down-regulates its gene promoter and that DNA binding activity of PARP is essential for its function in transcription.

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¹ The abbreviations used are: PARP, poly(ADP-ribose) polymerase; PARP-DBD, DNA-binding domain of PARP; AFM, atomic force microscopy; nt, nucleotide(s).

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The plasmid pPR-PARP was constructed by cloning the 5'-flanking region of the human PARP gene (from -899 to +156) fused to a chloramphenicol acetyltransferase reporter (20) into pcDNA 3.1 (Invitrogen) modified to remove the cytomegalovirus promoter. The 5'-deletion mutant of the PARP promoter (pΔPR-PARP) was generated as described previously (20). The expression plasmid pCD12 containing cDNA for human PARP has been described previously (21). pPARP-DBD was constructed by cloning the PCR-generated fragment of cDNA (22) for human PARP-DBD (amino acids 1–303) tagged at its carboxyl terminus with a sequence encoding four FLAG epitope tags into pcDNA 3.1. The integrity of all constructs was confirmed by sequence analysis.

DNA Heteroduplex Formation and Isolation—Heteroduplex formation between 301-bp *PvuII*-*PvuII* fragments of pUC8 and a similar fragment of pUC8F14C and isolation of the heteroduplex isomers were performed as described previously (23). Briefly, 10 μ l of hybridization mixture containing 1 pmol of each DNA fragment in 100 mM NaCl, 50 mM Tris-HCl, pH 7.9, 1 mM dithiothreitol, 10 mM MgCl₂ were incubated stepwise at 100 °C (1 min), 85 °C (10 min), and 70 °C (60 min) and then cooled to room temperature. Hybridization products were run in a 5% native polyacrylamide gel in 90 mM Tris borate (pH 8.3), 2.5 mM EDTA, and bands of heteroduplex fragments, which migrate slower than correctly annealed parental fragments (23), were excised. After an additional purification step using an UltraClean 15 DNA purification kit (MoBio, Solana Beach, CA), isolated heteroduplexes were resuspended in 60 μ l of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.8), and aliquots were taken for strand identification by sequencing and atomic force microscopy (AFM) analysis.

Supercoiled Topoisomer Preparation—Each of eight fractions of differently supercoiled DNA (topoisomers) was prepared by incubating 5 μ g of plasmid DNA purified by CsCl density gradient with 20 μ l of topoisomerase I-containing nuclear extract from HeLa cells (24) in the presence of appropriate concentrations of ethidium bromide (0–13 μ M) in 200 μ l of reaction buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.6) (25). Average superhelical densities of resultant topoisomer fractions were calculated as $\sigma = 10.5\tau/N$, where N is the number of base pairs in the plasmid, and τ is the number of superhelical turns determined by the band counting method after topoisomer separation in an agarose gel in the presence of chloroquine (26).

Assay for Base-unpaired Sites—The sequence of the 1.1-kb insert was analyzed for potential hairpin formation using MFOLD software.² The free energies of potential hairpins were calculated for single-stranded DNA at 37 °C in a solution containing 150 mM monovalent cation and 1 mM Mg²⁺. To detect unwound regions in supercoiled DNA, 1 μ g of each topoisomer prepared in a reaction with topoisomerase I was incubated on ice with 0.5 units of nuclease P1 (Invitrogen) in 10 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 50 mM NaCl at 37 °C for 10 min. The reaction was terminated by phenol/chloroform extraction, and DNA was recovered by ethanol precipitation. Following the *EcoRI* digestion to release a promoter-containing 1.1-kb insert, DNA was 3'-end-labeled using [α -³²P]dATP and the Klenow fragment of DNA polymerase from *Escherichia coli* (New England Biolabs). The resultant products were separated in their single-stranded forms on a 1.5% alkaline agarose gel in 50 mM NaOH (pH 12.5), 1 mM EDTA.

PARP Binding Reactions—A recombinant full-length human PARP (R&D Systems) was used in DNA binding reactions at a 4:1 molar ratio (protein to DNA) under the ionic conditions required for optimal PARP activity (4, 21). The heteroduplex DNA (23) containing stable 50-bp hairpin arms was used in PARP binding reactions. Parental duplexes (fragments of pUC8 and pUC8F14C plasmids) were used as controls in these experiments. For PARP binding reactions with the supercoiled or topologically relaxed DNA, plasmids were predigested with exonuclease III to exclude the presence of nicks in the DNA template (19). To analyze the interactions of PARP protein with the promoter region in supercoiled plasmids, bound PARP was cross-linked to DNA with 0.5% glutaraldehyde for 2 min at 37 °C, and the 1.1-kb *EcoRI*-*EcoRI* fragment containing the PARP promoter region was isolated and purified on Sephadex G25 spin columns equilibrated with the deposition buffer (10 mM HEPES, pH 7.3, 1 mM MgCl₂).

Chromatin Cross-linking and Immunoprecipitation—Ewing's sarcoma cells A4573 (kindly provided by Dr. T. Kinsella, University of Wisconsin, Madison) were grown and maintained in Eagle's minimal essential medium (Invitrogen). Formaldehyde (Fisher) was added di-

rectly to the cell culture medium to a final concentration of 1%, and fixation proceeded at 37 °C for 10 min as described in the ChIP assay protocol (Upstate Biotechnology). Immunoprecipitation was performed with rabbit polyclonal anti-PARP antibody (Cell Signaling Technology). Cross-links were reversed by heating to 65 °C for 4 h in the presence of 200 mM NaCl followed by PCR analysis of DNA for the detection of the PARP promoter sequences using upstream (5'-TGTCA ACCCA GAGAT GGCAT-3') and downstream (5'-AACTA CTCGG GAGGC TGAA-3') PCR primers designed according to the reported sequence data for the PARP 5'-region of the human PARP gene (27). Immunocapture of PARP from cross-linked chromatin was analyzed by immunoblotting with goat polyclonal anti-PARP antibody (1:1000, R&D Systems) as described previously (20).

Sample Preparation and Imaging with AFM—DNA samples or PARP-DNA binding reaction product in Mg²⁺-containing buffer (28) were deposited on an anatomically flat mica surface, allowed to adsorb for 1 min, rinsed with deionized water, and dried in a gentle nitrogen flow. The AFM images were obtained using a NanoScope IIIa instrument equipped with E-scanner (Digital Instruments, Santa Barbara, CA) operating in a tapping mode in air as described previously (28). The tapping frequency of the 125- μ m silicon cantilever was 300–400 KHz, and the nominal scanning rate was set at 1–2 Hz. No less than 150 uncomplexed DNA molecules and 100 PARP-DNA complexes were analyzed in each experiment.

Transfections and Reporter Assays—Mouse embryonic fibroblasts derived from both wild type and PARP knockout mice (29) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μ g/ml). DNA transfections were carried out using a SuperFect reagent (Qiagen) according to the protocol of the manufacturer. The total amount of DNA transfected was held constant with the pcDNA 3.1 (Invitrogen) empty vector. Chloramphenicol acetyltransferase reporter assays were performed as described previously (20) and normalized for transfection efficiency using a co-transfected pSV- β -gal vector (Promega) as an internal control. Each experiment was repeated at least three times, in duplicate, with independent plasmid preparations to assess reproducibility.

RESULTS AND DISCUSSION

PARP Binds to Hairpins in DNA Heteroduplexes—To investigate the interactions of PARP with DNA, we used AFM, which allows direct visualization of protein and DNA molecules at nanometer resolution (30–32). This approach was preferred to biochemical assays to address the hypothesis that PARP binding to DNA sites other than strand breaks was directed to single strand regions as observed in unwound structures in double-stranded DNA. Alternative DNA secondary structures are not thermodynamically stable in linear DNA fragments and, therefore, are not amenable to investigations of their functional transactions such as protein binding. Accordingly, our experimental approach used model heteroduplex constructs carrying stable DNA secondary structures. We used three-way junction heteroduplexes that contain 106-bp inverted repeats in one DNA strand (23) to form hairpin-like DNA structures (Fig. 1A). A representative AFM image shows that heteroduplex molecules have extrusions of the size expected for the 50-bp hairpin in the B conformation and bends at the junction (Fig. 1B).

After allowing full-length PARP protein to bind to the model hairpin-containing DNA, AFM images revealed a high incidence of DNA-protein complexes (~60% of all DNA molecules) that were divided into two types based on their locations in the heteroduplexes. In complexes of the first type, PARP associated primarily with DNA ends and less frequently dimerized heteroduplexes end-to-end (Fig. 1D) consistent with our previous observations that PARP can link DNA fragments into chain-like structures (28). The most striking observation was the occurrence of the second type, internal DNA-protein complexes (Fig. 1, C, D, and E). Proteins in these complexes resided at the junction site and were not observed in other internal regions of the long arms of the model DNA. Moreover, no internal PARP-DNA complexes were formed with control DNA duplexes

² Michael Zuker, Rensselaer Polytechnic Institute, bioinfo.math.rpi.edu/~zukerm/home.html.

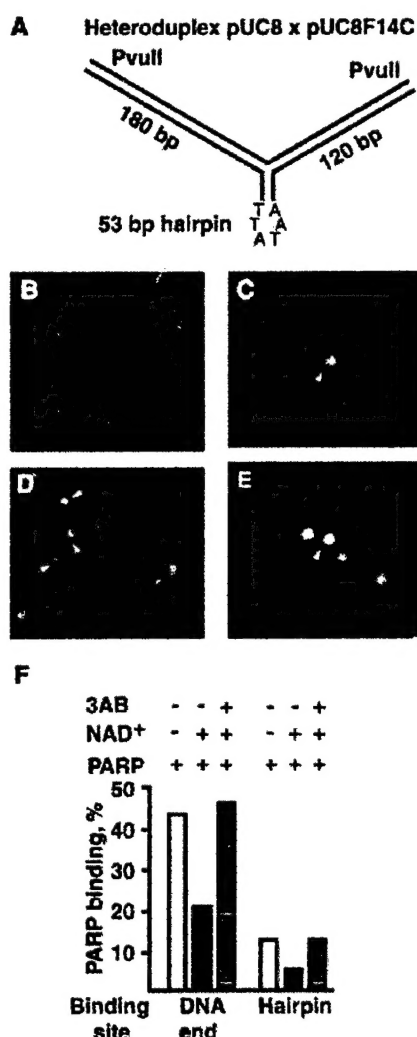


FIG. 1. Binding of recombinant PARP to three-way DNA junctions. A, schematic representation of heteroduplex DNA with an unpaired region at the apex of hairpin. B, AFM images of three-way DNA junctions containing a 50-bp hairpin (visible as the protrusion from the bend near the center of the molecule). C–E, representative AFM images of PARP-DNA complexes. End-bound (yellow arrows) and internally bound (white arrows) PARP molecules are indicated. Images show a 400- × 400-nm surface area. The color scale ranges from 0.0 to 4.0 nm (from dark to bright). F, the effects of NAD⁺ (0.1 mM) and 3-aminobenzamide (3AB) (1 mM) on the interaction of PARP with DNA ends and hairpins. PARP binding to DNA was calculated as the percentage of occurrence of the PARP-DNA complexes to the total number of heteroduplexes scored. Only unobstructed protein-DNA complexes were quantified. The total numbers of DNAs counted in each experiment ranged from 420 to 540 molecules.

(301-bp fragment of pUC8 and 401-bp fragment of pUC8F14C), thus indicating the specificity of PARP binding to hairpin-containing regions in double-stranded DNA. This finding presents a challenge to the generally accepted view that PARP binds only to strand breaks in DNA.

In the presence of NAD⁺, PARP bound to DNA strand breaks undergoes auto(ADP-ribosyl)ation, acquiring a high negative charge. Due to the charge repulsion the protein rapidly dissociates from DNA (4, 33, 34). Therefore, we next tested the ability of PARP to bind hairpin-containing DNA under conditions conducive to PARP automodification. Similar to our previous observations of PARP binding to DNA ends (28), NAD⁺ significantly decreased PARP affinity to the hairpins. Reversal of this effect was observed in the presence of 3-aminobenz-

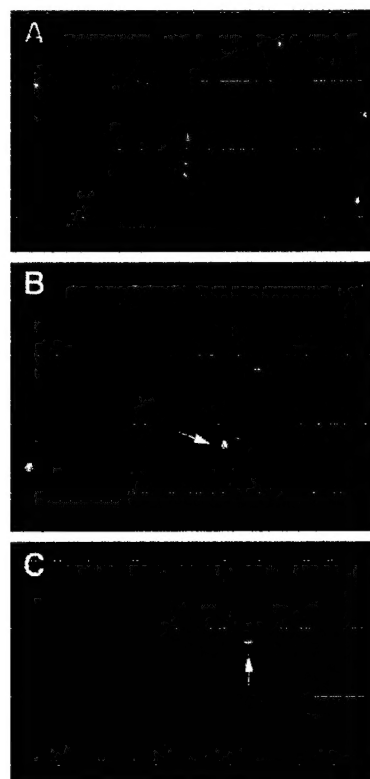


FIG. 2. Interaction of PARP protein with the 1.1-kb 5'-region of the PARP gene. A, binding of PARP to topologically relaxed pPR-PARP plasmid containing the PARP promoter region (from -899 to +156). B, binding of PARP to negatively supercoiled ($\sigma = -0.050$) pPR-PARP plasmid. C, AFM images of the PARP protein-promoter complexes. Bound PARP molecules were cross-linked to plasmid DNA with a superhelical density, σ of -0.050 , and the promoter-containing fragment (1.1 kb) was isolated for AFM examination. Representative images A and B show a 700- × 700-nm surface area, and image C shows an enlarged surface area (340 × 183 nm). Arrows (B and C) point to the PARP-DNA complex.

amide (Fig. 1F), a potent inhibitor of PARP catalytic activity. The relatively low yield of hairpin-protein complexes suggests that PARP has higher affinity to DNA ends than to hairpins in DNA fragments. These observations indicate that (i) PARP is capable of binding to certain secondary structures (e.g. hairpin-containing regions) in double-stranded DNA independently of the presence of DNA ends and (ii) NAD⁺-dependent automodification of PARP results in inhibition of its hairpin binding activity.

PARP Protein Binds to the 5'-Flanking Region of the PARP Gene—Accumulating evidence supports the involvement of DNA secondary structures such as hairpins and cruciforms in transcription (34–38). We reasoned that PARP affinity for stem-loops in DNA might influence regulation of transcription in undamaged cells by binding to such domains in promoter regions. To test this hypothesis, we investigated interaction of the PARP protein with the 5'-flanking region of the PARP gene (20). Structurally, the PARP gene promoter is TATA-deficient and G + C-rich, typical of promoters that contain dyad symmetry elements with high propensity to form secondary structures such as cruciforms (39). Secondary structures are favored when DNA is negatively supercoiled and are not thermodynamically stable in linear DNA fragments (40). Therefore, we examined the PARP interactions with supercoiled ($\sigma = -0.050$) and topologically relaxed ($\sigma = 0$) pPR-PARP plasmids (Fig. 2, A and B). PARP binding reactions were performed using the same DNA to protein molar ratio (4:1) as in experiments with

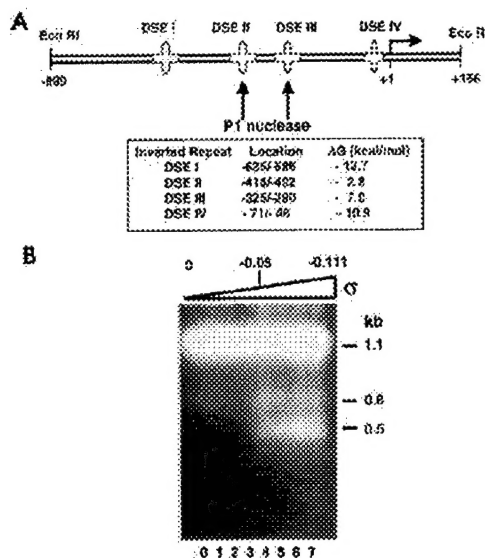


FIG. 3. Detection of P1 nuclease-sensitive sites in the PARP promoter. A, schematic representation of the human PARP promoter (from -899 to +1). The position of dyad symmetry elements (DSE) in the promoter sequence and the hairpin free energies calculated by the MFOLD program are indicated in the boxed area. Putative P1 nuclease-sensitive sites are shown with arrows. B, pPR-PARP topoisomers with superhelical density (σ) ranging from 0 to -0.111 were treated with P1 nuclease. The promoter-containing fragment (1.1 kb) was isolated and analyzed by alkaline agarose gel electrophoresis. The products of P1 nuclease digestion are denoted on the right. Topoisomer fractions 0-7 numbered at the bottom had the average σ of 0, -0.019, -0.031, -0.050, -0.065, -0.080, -0.094, and -0.111, respectively.

hairpin-containing DNA heteroduplexes. AFM imaging of DNA-protein interactions revealed that PARP is capable of binding to supercoiled plasmid in a DNA end-independent fashion. Further, a quantitative evaluation of the AFM images revealed a 3-4-fold higher yield of DNA-protein complexes on a supercoiled plasmid compared with topologically relaxed DNA. These data suggest that the preferential binding of PARP to supercoiled plasmid is attributable to the formation of recognition sites for PARP in torsionally stressed DNA.

To examine PARP protein-promoter interactions *in vitro*, bound proteins were cross-linked to superhelical plasmid ($\sigma = -0.050$) with 0.5% glutaraldehyde, and the 1.1-kb fragment containing the promoter region was isolated and examined by AFM. An average of 1.2 protein molecules were bound to the promoter-containing DNA duplex, indicating that PARP recognizes certain relatively infrequent sites in the promoter region (Fig. 2C). Although the PARP binding site(s) in its own promoter is yet to be identified, our data might conceivably reflect polymerase interaction with the regions of single-stranded character that can be formed in superhelical DNA. One potential option is the formation of cruciform-like structures since several imperfect inverted repeats have been identified in the promoter sequence by the computer algorithm MFOLD (Fig. 3A). In support of this, we observed the appearance of yet unidentified sites in the promoter region that are recognized by the single strand-specific nuclease P1. These sites are generated by unwinding torsional stress in supercoiled DNA with a threshold value of superhelical density $\sigma = -0.050$ (Fig. 3B) and were not detected in relaxed covalently closed plasmid DNA. Based on the size of P1 nuclease-generated fragments, the positions of the putative unwound sites correspond to imperfect inverted repeat (nt -325/-290) or an AT-rich region with dyad symmetry (nt -418/-403) in the PARP promoter sequences. Although these data suggest that the 5'-flanking region of the PARP gene has the ability to adopt unwound or

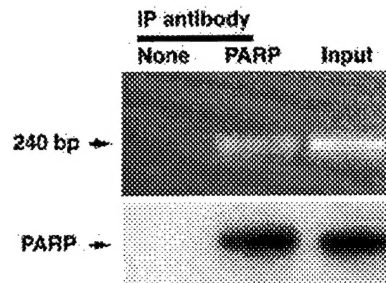


FIG. 4. PARP protein binds to the 5'-flanking region of the human PARP gene *in vivo*. Formaldehyde-cross-linked chromatin from asynchronously growing Ewing's sarcoma cells (cell line A4573) was immunoprecipitated using anti-PARP polyclonal antibody. A no-antibody immunoprecipitation was performed for a negative control (None). The input sample contains total chromatin before selection by immunoprecipitation. Top panel, immunoprecipitated DNA was analyzed by PCR using primers specific for the human PARP promoter. A 240-bp PCR fragment amplified from the PARP promoter sequence is shown. Bottom panel, immunoblotting analysis of PARP protein in cross-linked chromatin. IP, immunoprecipitation.

alternatively base-paired structures, further studies are required to assess functional transactions between PARP protein and such structures and to map PARP binding sites on the promoter.

To analyze the PARP protein-DNA interactions at the human PARP promoter *in vivo* we performed formaldehyde cross-linking and immunoprecipitation experiments. This approach permits analysis of DNA-binding proteins in eukaryotic cells under physiological conditions (41, 42). We observed that anti-PARP antibody effectively immunoprecipitated endogenous PARP protein and the 5'-flanking region of the PARP gene promoter (Fig. 4) from Ewing's sarcoma cells that constitutively express PARP protein (20). This observation indicates that PARP protein is recruited to the human PARP promoter sequences *in vivo*. It remains to be determined whether PARP protein binds to the promoter sequences as a monomer or forms a heterodimer with yet to be identified transcriptional regulator(s). In support of the latter possibility, the physical association of PARP with transcription factors TEF-1, B-MYB, and AP-2 and its involvement in the active transcriptional DNA-protein complex on *Reg* and *Pax-6* promoters have been recently demonstrated (11, 12, 17, 43, 44).

Transcriptional Autoregulation of the Human PARP Gene—The functional significance of PARP interactions with its gene promoter was evaluated by transient transfection assays using immortal fibroblasts (PARP^{-/-}) derived from PARP knock-out mice (29). We found that the transcriptional activity of the PARP promoter was 4-5-fold greater in PARP^{-/-} cells than in wild type (PARP^{+/+}) fibroblasts (Fig. 5A). Introduction of plasmid pCD12 carrying PARP cDNA into PARP^{-/-} cells conferred transcriptional down-regulation of the PARP gene promoter (Fig. 5B). These data are in accord with the previously reported observations that inducible PARP expression in PARP-producing cells also inhibited PARP promoter activity (45), thus suggesting intrinsic autoregulation of PARP expression. Next we observed that deletion of the -899 to -95 region from the PARP promoter sequences alleviated PARP-mediated transcriptional inhibition (Fig. 5C) thus indicating that at least some of the functional sites that are required for PARP-mediated down-regulation of transcription may reside upstream of the minimal PARP promoter (nt from -95 to +156). This suggestion agrees with our earlier observations that the PARP promoter region (nt -420/-290), harboring two putative unwound sites (at nt -418/-403 and -325/-290) (Fig. 3), is involved in negative control of the PARP promoter in cells

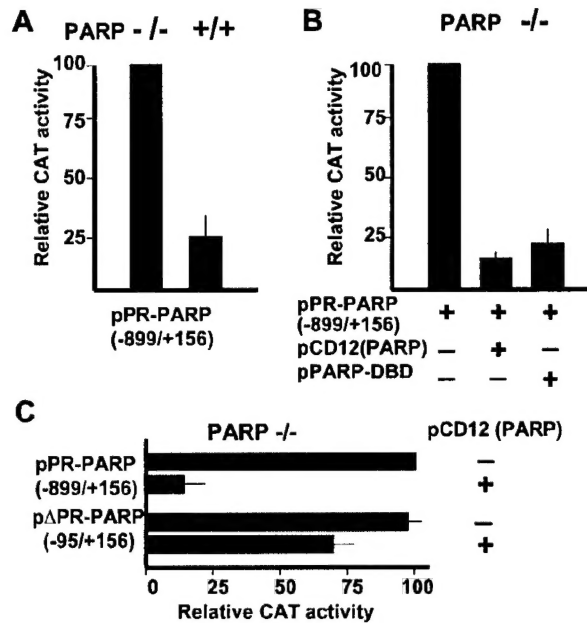


FIG. 5. PARP protein is a transcriptional repressor. A, PARP promoter transcriptional activity in wild type (PARP $^{+/+}$) and PARP $^{-/-}$ fibroblasts. B, expression of human PARP or its DNA-binding domain down-regulates promoter activity. PARP $^{-/-}$ cells were co-transfected with pPR-PARP and plasmids encoding for full-length PARP (pCD12) or its truncated mutant (pPARP-DBD). C, deletion of the distal region (-899 to -95) alleviates transcriptional repression by PARP protein. Vectors containing the PARP promoter (pPR-PARP) or its 5'-deletion mutant (pΔPR-PARP) were transiently co-transfected with the PARP-expressing vector into PARP $^{-/-}$ fibroblasts. Chloramphenicol acetyltransferase (CAT) activity of pPR-PARP in PARP $^{-/-}$ cells was arbitrarily taken as 100%. Means of triplicate experiments normalized by co-transfected β -galactosidase and S.D. are indicated.

naturally overexpressing PARP protein (20). To address the question whether catalytic activity of PARP is required for transcriptional down-regulation, the amino-terminal fragment of human PARP (amino acids 1–303) encompassing the region that encodes two zinc fingers of the enzyme and the proximal (amino acids 200–220) helix-turn-helix motif (22) was transiently expressed in PARP $^{-/-}$ cells. Co-transfection of the reporter gene (pPR-PARP) and a vector (pPARP-DBD) expressing a truncated PARP mutant (that contains the DNA-binding domain but lacks catalytic activity) resulted in transcriptional down-regulation of the PARP promoter in cells with a PARP-negative background (Fig. 5B), thus indicating that PARP-mediated inhibition of transcription was independent of PARP catalytic activity. Together these data demonstrate that PARP protein is a potent repressor of transcription when targeted to promoter and that its DNA binding activity is necessary and sufficient for transcriptional repression. However, we cannot rule out the possibility of cooperative interactions between PARP and other regulatory proteins for this repressive effect.

To conclude, the interactions of PARP protein with the promoter of its own gene result in suppression of transcription. PARP binding to secondary structures in DNA may reflect a potential mechanism by which it is recruited to the gene promoter. Furthermore, our data suggest that a hierarchy of PARP function may exist under which transcriptional repression may be abrogated in response to DNA damage due to a higher affinity of PARP for DNA breaks and its dissociation from DNA following protein automodification (Fig. 6). This concept integrates PARP functions in DNA repair (a nick-protection mechanism) (4, 33) and in transcriptional control of gene(s) involved in immediate cellular response to ionizing

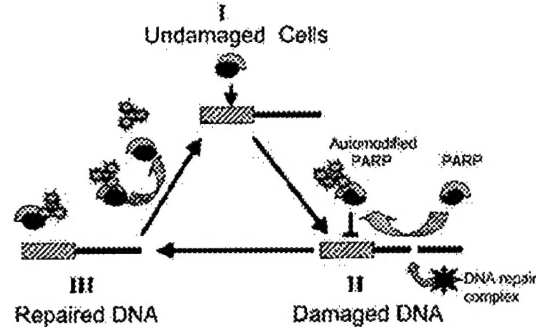


FIG. 6. A model for PARP-mediated regulation of transcription. I, in undamaged cells, unmodified PARP molecules bind to the DNA secondary structures within the gene promoter (denoted by a striped box). Such macromolecular interactions between PARP protein and a promoter region constitute a repressor function for PARP in transcription. II, in response to DNA damage, PARP binding to the DNA ends triggers its catalytic activity. Subsequent poly(ADP-ribosylation) of free and bound PARP in the presence of intracellular NAD $^{+}$ prevents its interaction with the promoter regions. This alleviates the PARP-mediated block on the promoter and up-regulates transcription of its own and other genes involved in the DNA damage response. III, the DNA binding activity of PARP is restored following DNA damage repair and the degradation of the ADP-ribose polymers by poly(ADP-ribose) glycohydrolase leading to reassembly of PARP-promoter complexes and inhibition of transcription.

radiation and DNA-damaging drugs. Although the evidence supporting such a mechanism is not yet available, it is conceivable that the sharing of components such as PARP by DNA repair and transcription allows both events to control cellular survival in response to ionizing radiation and DNA-damaging treatments. In support of this mechanism, PARP-dependent inhibition of transcription elongation by RNA polymerase II in undamaged cells and up-regulation of mRNA synthesis in response to DNA damage have been recently demonstrated both *in vitro* and *in vivo* (13). Studies testing this hypothesis are underway.

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